

TRANSLATOR'S DECLARATION

I, Janet Hope, BSc (Hons.), MIL., MITI., translator to Messrs. Taylor and Meyer of 20 Kingsmead Road, London, SW2 3JD, Great Britain, verify that I know well both the German and the English language, that I have prepared the attached English translation of 46 pages of a German Patent application in the German language with the title:

Neue für das otsA-Gen kodierende Nukleotidsequenzen

identified by the code number 010037 BT / IP at the upper left of each page and corresponding to client/matter number _____ of the law firm of _____

and that the attached English translation of this document is a true and correct translation of the document attached thereto to the best of my knowledge and belief.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of this document.

By:



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The attached papers are a true and accurate reproduction of the original documents for this patent application.

Munich, 22nd November 2001
On behalf of the President of the German
Patent and Trade Mark Office

(signature)

Wallner

New nucleotide sequences which code for the otsA gene

The invention provides nucleotide sequences from coryneform bacteria which code for the otsA gene and a process for the fermentative preparation of amino acids using bacteria in
5 which the otsA gene is attenuated.

Prior art

L-Amino acids, in particular L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal
10 nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve
15 the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to
20 the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these
25 microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce amino acids are obtained in this manner.

Methods of the recombinant DNA technique have also been
30 employed for some years for improving the strain of Corynebacterium strains which produce L-amino acid, by amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

Object of the invention

The inventors had the object of providing new measures for improved fermentative preparation of amino acids.

Description of the invention

- 5 Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-
10 isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Lysine
is particularly preferred.

When L-lysine or lysine are mentioned in the following, not only the bases but also the salts, such as e.g. lysine
15 monohydrochloride or lysine sulfate, are meant by this.

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the otsA gene, chosen from the group consisting of

- 20 a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

b) polynucleotide which codes for a polypeptide which
25 comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide which is complementary to the polynucleotides of a) or b),

- d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of trehalose 5 6-phosphate synthase.

The invention also provides the abovementioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

- (i) the nucleotide sequence, shown in SEQ ID No.1, or
10 (ii) at least one sequence which corresponds to sequence (i) within the degeneracy of the genetic code, or
(iii) at least one sequence which hybridizes with the sequences complementary to sequences (i) or (ii), and optionally
15 (iv) sense mutations of neutral function in (i) which do not modify the activity of the protein/polypeptide.

Finally, the invention also provides polynucleotides chosen from the group consisting of

- a) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ 20 ID No. 1 between positions 1 and 883,
b) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 884 and 2338,
25 c) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 2339 and 3010.

The invention also provides:

a polynucleotide, in particular DNA, which is capable of replication and comprises the nucleotide sequence as shown in SEQ ID No.1;

5 a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing parts of the polynucleotide according to the invention, but at least 15 successive nucleotides of the sequence claimed,

10 and coryneform bacteria in which the otsA gene is attenuated, in particular by an insertion or deletion.

The invention also provides polynucleotides, which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a 15 corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide 20 sequence mentioned.

Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for 25 trehalose 6-phosphate synthase or to isolate those nucleic acids or polynucleotides or genes which have a high similarity with the sequence of the otsA gene. They are also suitable for incorporation into so-called "arrays", "micro arrays" or "DNA chips" in order to detect and 30 determine the corresponding polynucleotides.

Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for trehalose 6-

phosphate synthase can be prepared by the polymerase chain reaction (PCR).

Such oligonucleotides which serve as probes or primers comprise at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24, very particularly preferably at least 15, 16, 17, 18 or 19 successive nucleotides.

Oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 or at least 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides are also suitable.

10 Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides are optionally also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these 15 to be non-modified RNA or DNA or modified RNA or DNA.

The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly 20 preferably at least 86% to 90% and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

25 "Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of trehalose 6-phosphate 30 synthase, and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90% and very particularly preferably at least

91%, 93%, 95%, 97% or 99% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

The invention furthermore relates to a process for the fermentative preparation of amino acids chosen from the
5 group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine using coryneform bacteria which in particular
10 already produce amino acids and in which the nucleotide sequences which code for the otsA gene are attenuated, in particular eliminated or expressed at a low level.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of
15 one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme or protein with a low activity or inactivates the corresponding gene or enzyme (protein) and
20 optionally combining these measures.

The microorganisms provided by the present invention can prepare amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of
25 coryneform bacteria, in particular of the genus Corynebacterium. Of the genus Corynebacterium, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

30 Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium melassecola ATCC17965
5 Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared
10 therefrom, such as, for example, the L-lysine-producing
strains

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
15 Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum DM58-1
Corynebacterium glutamicum DG52-5
Corynebacterium glutamicum DSM5715 and
20 Corynebacterium glutamicum DSM12866.

The new otsA gene from *C. glutamicum* which codes for the enzyme trehalose 6-phosphate synthase (EC 2.4.1.15) has been isolated.

To isolate the otsA gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in *Escherichia coli* (*E. coli*). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the *E. coli* K-12 strain W3110 set up in λ vectors by Kohara et al.

(Cell 50, 495-508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, 5 Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326 (1992)) in turn describe a gene library of *C. glutamicum* 10 ATCC13032 using the cosmid pHG79 (Hohn and Collins, 1980, Gene 11, 291-298).

To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, 1979, Life Sciences, 25, 807-818) or pUC9 (Vieira et al., 15 1982, Gene, 19:259-268). Suitable hosts are, in particular, those *E. coli* strains which are restriction- and recombination-defective, such as, for example, the strain DH5 α mcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 20 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids or other λ vectors can then in turn be subcloned and subsequently sequenced in the usual vectors which are suitable for DNA sequencing, such as is described e. g. by Sanger et al. (Proceedings of the National Academy 25 of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-30 232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of *C. glutamicum* which codes for the otsA gene and which, as SEQ ID No. 1, is a constituent of

the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the 5 otsA gene product is shown in SEQ ID No. 2. It is known that enzymes endogenous in the host can split off the N-terminal amino acid methionine or formylmethionine of the protein formed.

Coding DNA sequences which result from SEQ ID No. 1 by the 10 degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or 15 of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. Such mutations are also called, inter alia, neutral substitutions. It is 20 furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in 25 O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks 30 of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result 35 from SEQ ID No. 1 are a constituent of the invention. Such

oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in
5 the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)). The hybridization takes place under stringent conditions, that
10 is to say only hybrids in which the probe and target sequence, i. e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer
15 composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

20 A 5x SSC buffer at a temperature of approx. 50°C - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are
25 removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995), a temperature of
30 approx. 50°C - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% or at least 96% to 99% identical to the sequence of the probe employed can
35 be isolated by increasing the hybridization temperature

stepwise from 50°C to 68°C in steps of approx. 1 - 2°C. It is also possible to isolate polynucleotide fragments which are completely identical to the sequence of the probe employed. Further instructions on hybridization are 5 obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by 10 the expert, inter alia, in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has been found that coryneform bacteria produce amino 15 acids in an improved manner after attenuation of the otsA gene.

To achieve an attenuation, either the expression of the otsA gene or the catalytic/regulatory properties of the enzyme protein can be reduced or eliminated. The two 20 measures can optionally be combined.

The reduction in gene expression can take place by suitable culturing or by genetic modification (mutation) of the signal structures of gene expression. Signal structures of gene expression are, for example, repressor genes, 25 activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The expert can find information on this e.g. in the patent application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss 30 (Nucleic Acids Research 26: 3548 (1998)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)), in Pátek et al. (Microbiology 142: 1297 (1996)), Vasicova et al. (Journal of Bacteriology 181: 6188 (1999)) and in known textbooks of genetics and molecular biology, such as

e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

5 Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art; examples which may be mentioned are the works by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and
10 Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydrolase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms", Reports from the Jülich Research Centre, Jül-2906, ISSN09442952, Jülich, Germany, 1994). Summarizing
15 descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Possible mutations are transitions, transversions,
20 insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair (bp) in a gene lead to frame shift mutations, as a consequence of
25 which incorrect amino acids are incorporated or translation is interrupted prematurely. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known textbooks of genetics and
30 molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer
35 Verlag, Stuttgart, 1986).

A common method of mutating genes of *C. glutamicum* is the method of "gene disruption" and "gene replacement" described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

5 In the method of gene disruption a central part of the coding region of the gene of interest is cloned in a plasmid vector which can replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791
10 10 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-
15 84; US Patent 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516). The plasmid vector which contains the central part of the coding region
20 of the gene is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for
25 example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross-over" event,
30 the coding region of the gene in question is interrupted by the vector sequence and two incomplete alleles are obtained, one lacking the 3' end and one lacking the 5' end. This method has been used, for example, by Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580
35 (1994)) to eliminate the recA gene of *C. glutamicum*.

In the method of "gene replacement", a mutation, such as e.g. a deletion, insertion or base exchange, is established in vitro in the gene of interest. The allele prepared is in turn cloned in a vector which is not replicative for C.

5 glutamicum and this is then transferred into the desired host of C. glutamicum by transformation or conjugation. After homologous recombination by means of a first "cross-over" event which effects integration and a suitable second "cross-over" event which effects excision in the target 10 gene or in the target sequence, the incorporation of the mutation or of the allele is achieved. This method was used, for example, by Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998)) to eliminate the pyc gene of C. glutamicum by a deletion.

15 A deletion, insertion or a base exchange can be incorporated into the otsA gene in this manner.

In addition, it may be advantageous for the production of L-amino acids to enhance, in particular over-express, one or more enzymes of the particular biosynthesis pathway, of 20 glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the attenuation of the otsA gene.

The term "enhancement" in this connection describes the 25 increase in the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or of the genes or alleles, using a potent promoter or using a gene or allele which codes for a 30 corresponding enzyme having a high activity, and optionally combining these measures.

Thus, for the preparation of L-lysine, in addition to the attenuation of the otsA gene at the same time one or more of the genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
 - the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 5 174:6076-6086),
 - the eno gene which codes for enolase (DE: 19947791.4),
 - the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
 - the pgk gene which codes for 3-phosphoglycerate kinase 10 (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
 - the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
 - the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
 - 15 • the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
 - the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512; EP-B-0387527; 20 EP-A-0699759; WO 00/63388),
 - the lysE gene which codes for lysine export (DE-A-195 48 222),
 - the zwal gene which codes for the Zwal protein (DE: 19959328.0, DSM 13115)
- 25 can be enhanced, in particular over-expressed.

It may be furthermore advantageous for the production of L-lysine, in addition to the attenuation of the otsA gene, at the same time for one or more of the genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM 13047),
 - the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- 5 • the poxB gene which codes for pyruvate oxidase (DE:1995 1975.7, DSM 13114),
- the zwa2 gene which codes for the Zwa2 protein (DE: 19959327.2, DSM 13113),
 - the fda gene which codes for fructose 1,6-bisphosphate aldolase (Accession No. X17313; von der Osten et al., Molecular Microbiology 3 (11), 1625-1637 (1989)),
- 10 • the hom gene which codes for homoserine dehydrogenase (EP-A-0131171),
- the thrB gene which codes for homoserine kinase (Peoples, O.W., et al., Molecular Microbiology 2 (1988): 63 - 72) and
 - the panD gene which codes for aspartate decarboxylase (EP-A-1006192) and

20 to be attenuated, in particular for the expression thereof to be reduced.

The attenuation of homoserine dehydrogenase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-valine for L-alanine, L-glycine or L-leucine in position 59 of the enzyme protein, by exchange of L-valine by L-isoleucine, L-valine or L-leucine in position 104 of the enzyme protein and/or by exchange of L-asparagine by L-threonine or L-serine in positioin 118 of the enzyme protein.

25 The attenuation of homoserine kinase can also be achieved, inter alia, by amino acid exchanges, such as, for example,

by exchange of L-alanine for L-valine, L-glycine or L-leucine in position 133 of the enzyme protein and/or by exchange of L-proline by L-threonine, L-isoleucine or L-serine in position 138 of the enzyme protein.

- 5 The attenuation of aspartate decarboxylase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchanges of L-alanine for L-glycine, L-valine or L-isoleucine in position 36 of the enzyme protein.
- 10 In addition to the attenuation of the otsA gene it may furthermore be advantageous for the production of amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, 15 Vanek (eds.), Academic Press, London, UK, 1982).

The invention also provides the microorganisms prepared according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed 20 batch process (repetitive feed process) for the purpose of production of L-amino acids. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 25 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions 30 of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

- Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for example, soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as, 5 for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.
- 10 Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be 15 used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.
- Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus.
- 20 The culture medium must furthermore comprise salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances.
- 25 Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.
- Basic compounds, such as sodium hydroxide, potassium 30 hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.
- Antifoams, such as, for example, fatty acid polyglycol esters, can be employed to control the development of foam.
- 35 Suitable substances having a selective action, such as, for

example, antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as, for example, air, are introduced into the culture. The 5 temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

Methods for the determination of L-amino acids are known 10 from the prior art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography with subsequent ninhydrin derivatization, or it can be carried out by reversed phase HPLC, for example 15 as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention is used for the fermentative preparation of amino acids, in particular L-lysine.

20 The following microorganism was deposited on 08.02.2001 as a pure culture at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- 25 • Corynebacterium glutamicum strain DSM5715ΔotsA as DSM 14041.

The present invention is explained in more detail in the following with the aid of embodiment examples.

30 The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA).

Methods for transformation of *Escherichia coli* are also described in this handbook.

The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et al.

Example 1

Preparation of a genomic cosmid gene library from *C. glutamicum* ATCC 13032

- Chromosomal DNA from *C. glutamicum* ATCC 13032 is isolated
10 as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments are
15 dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987), Proceedings of the National Academy of Sciences, USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description
20 SuperCos1 Cosmid Vector Kit, Code no. 251301) is cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.
25 The cosmid DNA is then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner is mixed with the treated ATCC13032 DNA and the batch is treated with T4 DNA ligase (Amersham
30 Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture is then packed in phages with the aid of Gigapack II XL Packing

Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acids Res. 16:1563-1575) the cells are taken 5 up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library are carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 10 1955, Virology, 1:190) + 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones are selected.

Example 2

Isolation and sequencing of the otsA gene

15 The cosmid DNA of an individual colony is isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product 20 Description Sau3AI, Product No. 27-0913-02). The DNA fragments are dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid 25 fragments in the size range of 1500 to 2000 bp are isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description 30 Zero Background Cloning Kit, Product No. K2500-01) is cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid

fragments in the sequencing vector pZero-1 is carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture is then electroporated (Tauch et al. 1994, FEMS Microbiol. Letters, 123:343-7) into the *E. coli* strain DH5 α mcr (Grant, 1990, Proceedings of the National Academy of Sciences, U.S.A., 87:4645-4649). Letters, 123:343-7) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

The plasmid preparation of the recombinant clones is carried out with a Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing is carried out by the dideoxy chain-stopping method of Sanger et al. (1977, Proceedings of the National Academies of Sciences, U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction are carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained are then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives are assembled to a continuous contig. The computer-assisted coding region analysis is prepared with the XNIP program (Staden, 1986, Nucleic Acids Research 14:217-231).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence shows an open reading

frame of 1485 bp, which is called the otsA gene. The otsA gene codes for a polypeptide of 485 amino acids.

Example 3

Construction of the vector pK19mobsacBΔotsA for deletion of
5 the otsA gene

3.1. Cloning of the otsA gene in the vector pUC18

For this, chromosomal DNA is isolated from the strain ATCC13032 by the method of Tauch et al. (1995, Plasmid 33:168-179). On the basis of the sequence of the otsA gene 10 known for C. glutamicum from example 2, the oligonucleotides described below are chosen for generation of the otsA deletion allele (see also SEQ ID No. 3 and SEQ ID No.4):

otsA fwd:

5'- CAC CTA TTC TAA GGA CTT CTT CGA -3'

15 otsA rev:

5'-ACC AAC CAG GTG GAA TCT GTC A-3'

The primers shown are synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR protocols. A 20 Guide to Methods and Applications, 1990, Academic Press) with the Taq-polymerase from Boehringer Mannheim (Germany, Product Description Taq DNA polymerase, Product No. 1 146 165). With the aid of the polymerase chain reaction, the primers allow amplification of a DNA fragment approx. 25 1.8 kb in size. The product amplified in this way is tested electrophoretically in a 0.8% agarose gel.

The PCR product obtained is then cloned in the vector pUC18 (Amersham Pharmacia Biotech, Cat. No. 27-4949-01) with the Sure Clone Ligation Kit from Amersham Pharmacia Biotech 30 (Freiburg, Germany) in accordance with the manufacturer's

instructions. The vector pUC18 was linearized beforehand with the restriction enzyme SmaI.

The E.coli strain DH5 α mcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) is then 5 electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, IRL-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation 10 batch on LB agar(Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which has been supplemented with 25 mg/l ampicillin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by 15 restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid is called pUC18otsA and is shown in figure 1.

3.2. Introduction of a deletion into the cloned otsA gene fragment

20 From the plasmid pUC18otsA, a fragment 213 bp in size is excised from the central region of the otsA gene with the restriction enzymes PflMI and HpaI. The 3' overhangs formed from the PflMI digestion are removed with T4 DNA polymerase (Amersham Pharmacia Biotech, Freiburg, Germany; Code No. 25 E2040Y) in accordance with the manufacturer's instructions. The residual vector is subjected to autoligation with T4 DNA ligase (Amersham Pharmacia Biotech, Freiburg, Germany; Code No. 27-0870-04) in accordance with the manufacturer's instructions and the ligation batch is electroporated 30 (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) in the E. coli strain DH5 α (Hanahan, In: DNA cloning. A Practical Approach. Vol. I, IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Lennox,

1955, Virology, 1:190) with 25 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106,

5 Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and cleaved with the restriction enzyme EcoRI to check the plasmid by subsequent agarose gel electrophoresis. The resulting plasmid is called pUC18ΔotsA.

10 3.3. Construction of the replacement vector pK19mobsacΔotsA

The otsA deletion allele is isolated by complete cleavage of the vector pUC18ΔotsA, obtained in example 3.2, with the restriction enzymes SacI/XbaI. After separation in an agarose gel (0.8%), the otsAdel fragment approx. 1.6 kb in

15 size is isolated from the agarose gel with the aid of the Qiagenquick Gel Extraction Kit (Qiagen, Hilden, Germany). The 5' and 3' overhangs formed by the restriction digestion are removed with T4 DNA polymerase (Amersham Pharmacia Biotech, Freiburg, Germany; Code No. E2040Y) in accordance
20 with the manufacturer's instructions.

The otsA deletion allele treated in this way is employed for ligation with the mobilizable cloning vector pK19mobsacB (Schäfer et al., Gene 14: 69-73 (1994)). This was cleaved open beforehand with the restriction enzyme

25 SmaI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product No. 1758250). The vector DNA is mixed with the otsA deletion allele and the mixture is treated with T4 DNA ligase (Amersham- Pharmacia, Freiburg, Germany).

30 The E.coli strain DH5αmcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) is then electroporated with the ligation batch (Hanahan, In. DNA cloning. A practical approach. Vol.1. ILR-Press, Cold Spring Habor, New York, 1989). Selection of plasmid-

carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which has been supplemented with 25 mg/l kanamycin.

5 Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and the cloned otsA deletion allele is verified by means of sequencing by MWG Biotech (Ebersberg, Germany). The plasmid is called pK19mobsacBΔotsA and is shown in figure 2.

10 Example 4

Deletion mutagenesis of the otsA gene in the *C. glutamicum* strain DSM 5715

The vector pK19mobsacBΔotsA mentioned in example 3.3 is elecroporated by the elecroporation method of Tauch et

15 al. (1989 FEMS Microbiology Letters 123: 343-347) in *Corynebacterium glutamicum* DSM5715. The vector cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome.

Selection of clones with integrated pK19mobsacBΔotsA takes 20 place by plating out the elecroporation batch on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which was supplemented with 15 mg/l kanamycin. Incubation is carried 25 out for 2 days at 33°C.

Clones which have grown on are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 16 hours at 33°C.

To achieve excision of the plasmid together with the complete chromosomal copy of the otsA gene, the clones are

30 then grown on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989) with 10% sucrose. The plasmid pK19mobsacB contains a copy of the sacB gene, which converts sucrose into levan

sucrase, which is toxic to *C. glutamicum*. Only those clones in which the pK19mobsacB Δ otsA integrated has been excised again therefore grow on LB agar with sucrose. In the excision, together with the plasmid either the complete 5 chromosomal copy of the otsA gene can be excised, or the incomplete copy with the internal deletion. To demonstrate that the incomplete copy of otsA has remained in the chromosome, the plasmid pK9mobsacB Δ otsA is marked by the method of "The DIG System Users Guide for Filter 10 Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) using the Dig hybridization kit from Boehringer. Chromosomal DNA of a potential deletion mutant is isolated by the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)) and in each case cleaved with the 15 restriction enzymes EcoRI and PstI in separate batches. The fragments formed are separated by agarose gel electrophoresis and hybridized at 68°C with the Dig hybridization kit from Boehringer. With the aid of the fragments formed, it can be shown that the strain DSM5715 20 has lost its complete copy of the otsA gene and instead has only the copy with the deletion.

The strain is called *C. glutamicum* DSM5715 Δ otsA and deposited as a pure culture on 08.02.2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = 25 German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) as DSM 14041 in accordance with the Budapest Treaty.

Example 5

Preparation of lysine

30 The *C. glutamicum* strain DSM5715 Δ otsA obtained in example 4 is cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant is determined.

For this, the strain is first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l) for 24 hours at 33°C. Starting from this agar plate culture, a preculture is seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII is used as the medium for the preculture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Sucrose (autoclaved separately)	2% (w/v)

The pH is brought to pH 7.4

Kanamycin (25 mg/l) is added to this. The preculture is incubated for 16 hours at 33°C at 240 rpm on a shaking 10 machine. A main culture is seeded from this preculture such that the initial OD (660 nm) of the main culture is 0.1 OD. The medium Cg XII (Keilhauer et al. 1993, Journal of Bacteriology 175:5595-5603) with addition of 0.1 g/l leucine is used for the main culture.

15 Medium Cg XII

MOPS (morpholinopropanesulfonic acid)	42 g/l
Urea	5 g/l
Salts:	
(NH ₄) ₂ SO ₄	20 g/l
KH ₂ PO ₄	1 g/l
K ₂ HPO ₄	1 g/l

MgSO ₄ * 7 H ₂ O	0.25 g/l
CaCl ₂ * 2 H ₂ O	10 mg/l
FeSO ₄ * 7 H ₂ O	10 mg/l
MnSO ₄ * H ₂ O	10 mg/l
ZnSO ₄ * 7 H ₂ O	1 mg/l
CuSO ₄	0.2 mg/l
NiCl ₂	0.02 mg/l
Biotin (sterile-filtered)	0.3 mg/l
Leucine (sterile-filtered)	0.1 g/l
Protocatechuic acid (sterile-filtered)	0.03 mg/l
Sucrose (autoclaved separately)	6% (w/v)

MOPS and the salt solution are brought to pH 7 and autoclaved. The sterile substrate and vitamin solutions are then added.

5 Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) is added. Culturing is carried out at 33°C and 80% atmospheric humidity.

10 After 73 hours, the OD is determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed is determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in table 1.

Table 1

Strain	OD (660 nm)	Lysine HCl mM
DSM5715	8.2	39
DSM5715 Δ otsA	8.4	52

The following figures are attached:

- Figure 1: Plasmid pUC18otsA
- 5 • Figure 2: Plasmid pK19mobsacB Δ otsA

The base pair numbers stated are approx. values obtained in the context of reproducibility.

The abbreviations and designations used have the following meaning:

- lacZ': 5' terminus of the lacZ α gene fragment
 'lacZ: 3' terminus of the lacZ α gene fragment
 otsA: otsA Gene
 Amp: Ampicillin resistance gene
 oriV: ColE1-similar origin from pMB1
 RP4mob: RP4 mobilization site
 Kan: Kanamycin resistance gene
 otsA': 5' terminal fragment of the pck gene
 'otsA: 3' terminal fragment of the pck gene
 sacB: The sacB gene which codes for the protein

levan sucrose

EcoRI: Cleavage site of the restriction enzyme EcoRI

HpaI: Cleavage site of the restriction enzyme HpaI

PflMI: Cleavage site of the restriction enzyme PflMI

PstI: Cleavage site of the restriction enzyme PstI

SacI: Cleavage site of the restriction enzyme SacI

XbaI: Cleavage site of the restriction enzyme XbaI

SEQUENCE PROTOCOL

<110> Degussa AG

5 <120> New nucleotide sequences which code for the otsA gene

<130> 010037 BT

<140>

10 <141>

<160> 4

<170> PatentIn Ver. 2.1

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<211> 3010

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<213> Corynebacterium glutamicum

20 <220>

<221> CDS

<222> (884)..(2338)

<223> otsA gene

25 <400> 1

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ggcccccgtct ctgccgtgc gattgctgca acagcagttg gtttcactgg tgggttgctt 120

30 gcccgtcgat tcttgattcc accgttgatt gtggcgattt ccggcatcac accaatgctt 180

ccaggtcttag caatttaccg cggaaatgtac gccaccctga atgatcaaac actcatgggt 240

35 ttcaccaaca ttgcgggtgc ttttagccact gcttcatcac ttgccgttgc cgtggtttg 300

ggtgagtggaa ttgcccccgag gctacgtcgt ccaccacgct tcaacccata ccgtgcattt 360

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aaacgtccaa agactaatca gagattcggt aataaaaaggtaaaaatcaac ctgcttaggc 480

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ttctgaacac gaggaagctc agccaaagaa ggctacaaag cggactcgta aggaccacgc 780

taagaaggct gctgctaaga aaacgaccaa gaagaccact aagaaaacta ctaaaaagac 840

55 caccgcaaag aagaccacaa agaagtctta agccggatct tat atg gat gat tcc 895

Met Asp Asp Ser

	aat agc ttt gta gtt gtt gct aac cgt ctg cca gtg gat atg act gtc	943
	Asn Ser Phe Val Val Val Ala Asn Arg Leu Pro Val Asp Met Thr Val	
5	5 10 15 20	
	cac cca gat ggt agc tat agc atc tcc ccc agc ccc ggt ggc ctt gtc	991
	His Pro Asp Gly Ser Tyr Ser Ile Ser Pro Ser Pro Gly Gly Leu Val	
	25 30 35	
10	acg ggg ctt tcc ccc gtt ctg gaa caa cat cgt gga tgt tgg gtc gga	1039
	Thr Gly Leu Ser Pro Val Leu Glu Gln His Arg Gly Cys Trp Val Gly	
	40 45 50	
15	tgg cct gga act gta gat gtt gca ccc gaa cca ttt cga aca gat acg	1087
	Trp Pro Gly Thr Val Asp Val Ala Pro Glu Pro Phe Arg Thr Asp Thr	
	55 60 65	
20	ggt gtt ttg ctg cac cct gtt gtc ctc act gca agt gac tat gaa ggc	1135
	Gly Val Leu Leu His Pro Val Val Leu Thr Ala Ser Asp Tyr Glu Gly	
	70 75 80	
	ttc tac gag ggc ttt tca aac gca acg ctg tgg cct ctt ttc cac gat	1183
	Phe Tyr Glu Gly Phe Ser Asn Ala Thr Leu Trp Pro Leu Phe His Asp	
	85 90 95 100	
25	ctg att gtt act ccg gtg tac aac acc gat tgg tgg cat gcg ttt cgg	1231
	Leu Ile Val Thr Pro Val Tyr Asn Thr Asp Trp Trp His Ala Phe Arg	
	105 110 115	
30	gag gta aac ctc aag ttc gct gaa gcc gtg agc caa gtg gcg gca cac	1279
	Glu Val Asn Leu Lys Phe Ala Glu Ala Val Ser Gln Val Ala Ala His	
	120 125 130	
35	ggt gcc act gtg tgg gtg cag gac tat cag ctg ttg ctg gtt cct ggc	1327
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	Ile Leu Arg Gln Met Arg Pro Asp Leu Lys Ile Gly Phe Phe Leu His	
	150 155 160	
	att ccc ttc cct tcc cct gat ctg ttc cgt cag ctg ccg tgg cgt gaa	1423
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	Glu Ile Val Arg Gly Met Leu Gly Ala Asp Leu Val Gly Phe His Leu	
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	215 220 225	
	gaa gca ttg gtg cgt gag att ggc gct cat gtt gaa acc gct gac gga	1615
	Glu Ala Leu Val Arg Glu Ile Gly Ala His Val Glu Thr Ala Asp Gly	
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20	ggc gcg ttg gag gcc gac aaa gct gtg ttg ctg cag gtc gcg acg cct Gly Ala Leu Glu Ala Asp Lys Ala Val Leu Leu Gln Val Ala Thr Pro 310	315	320		1855
25	tcg cgt gag cgc att gat cac tat cgt gtg tcg cgt tcg cag gtc gag Ser Arg Glu Arg Ile Asp His Tyr Arg Val Ser Arg Ser Gln Val Glu 325	330	335	340	1903
	gaa gcc gtc ggc cgt atc aat ggt cgt ttc ggt cgc atg ggg cgt ccc Glu Ala Val Gly Arg Ile Asn Gly Arg Phe Gly Arg Met Gly Arg Pro 345	350	355		1951
30	gtg gtg cat tat cta cac agg tca ttg agc aaa aat gat ctc cag gtg Val Val His Tyr Leu His Arg Ser Leu Ser Lys Asn Asp Leu Gln Val 360	365	370		1999
35	ctg tat acc gca gcc gat gtc atg ctg gtt acg cct ttt aaa gac ggt Leu Tyr Thr Ala Ala Asp Val Met Leu Val Thr Pro Phe Lys Asp Gly 375	380	385		2047
40	atg aac ttg gtg gct aaa gaa ttc gtg gcc aac cac cgc gac ggc act Met Asn Leu Val Ala Lys Glu Phe Val Ala Asn His Arg Asp Gly Thr 390	395	400		2095
	ggt gct ttg gtg ctg tcc gaa ttt gcc ggc gcg gcc act gag ctg acc Gly Ala Leu Val Leu Ser Glu Phe Ala Gly Ala Ala Thr Glu Leu Thr 405	410	415	420	2143
45	ggt gcg tat tta tgc aac cca ttt gat gtg gaa tcc atc aaa cgg caa Gly Ala Tyr Leu Cys Asn Pro Phe Asp Val Glu Ser Ile Lys Arg Gln 425	430	435		2191
50	atg gtg gca gct gtc cat gat ttg aag cac aat ccg gaa tct gcg gca Met Val Ala Ala Val His Asp Leu Lys His Asn Pro Glu Ser Ala Ala 440	445	450		2239
55	acg cga atg aaa acg aac agc gag cag gtc tat acc cac gac gtc aac Thr Arg Met Lys Thr Asn Ser Glu Gln Val Tyr Thr His Asp Val Asn 455	460	465		2287

gtg tgg gct aat agt ttc ctg gat tgt ttg gca cag tcg gga gaa aac 2335
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 470 475 480

5. tca tgaaccgcgc acgaatcgcg accataggcg ttcttccgct tgctttactg 2388
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 485

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35 <213> Corynebacterium glutamicum

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 1 5 10 15

40 Asp Met Thr Val His Pro Asp Gly Ser Tyr Ser Ile Ser Pro Ser Pro
 20 25 30

45 Gly Gly Leu Val Thr Gly Leu Ser Pro Val Leu Glu Gln His Arg Gly
 35 40 45

Cys Trp Val Gly Trp Pro Gly Thr Val Asp Val Ala Pro Glu Pro Phe
 50 55 60

50 Arg Thr Asp Thr Gly Val Leu Leu His Pro Val Val Leu Thr Ala Ser
 65 70 75 80

Asp Tyr Glu Gly Phe Tyr Glu Gly Phe Ser Asn Ala Thr Leu Trp Pro
 85 90 95

55 Leu Phe His Asp Leu Ile Val Thr Pro Val Tyr Asn Thr Asp Trp Trp
 100 105 110

	His Ala Phe Arg Glu Val Asn Leu Lys Phe Ala Glu Ala Val Ser Gln			
	115	120	125	
	Val Ala Ala His Gly Ala Thr Val Trp Val Gln Asp Tyr Gln Leu Leu			
5	130	135	140	
	Leu Val Pro Gly Ile Leu Arg Gln Met Arg Pro Asp Leu Lys Ile Gly			
	145	150	155	160
10	Phe Phe Leu His Ile Pro Phe Pro Ser Pro Asp Leu Phe Arg Gln Leu			
	165	170	175	
	Pro Trp Arg Glu Glu Ile Val Arg Gly Met Leu Gly Ala Asp Leu Val			
	180	185	190	
15	Gly Phe His Leu Val Gln Asn Ala Glu Asn Phe Leu Ala Leu Thr Gln			
	195	200	205	
	Gln Val Ala Gly Thr Ala Gly Ser His Val Gly Gln Pro Asp Thr Leu			
20	210	215	220	
	Gln Val Ser Gly Glu Ala Leu Val Arg Glu Ile Gly Ala His Val Glu			
	225	230	235	240
25	Thr Ala Asp Gly Arg Arg Val Ser Val Gly Ala Phe Pro Ile Ser Ile			
	245	250	255	
	Asp Val Glu Met Phe Gly Glu Ala Ser Lys Ser Ala Val Leu Asp Leu			
	260	265	270	
30	Leu Lys Thr Leu Asp Glu Pro Glu Thr Val Phe Leu Gly Val Asp Arg			
	275	280	285	
	Leu Asp Tyr Thr Lys Gly Ile Leu Gln Arg Leu Leu Ala Phe Glu Glu			
35	290	295	300	
	Leu Leu Glu Ser Gly Ala Leu Glu Ala Asp Lys Ala Val Leu Leu Gln			
	305	310	315	320
40	Val Ala Thr Pro Ser Arg Glu Arg Ile Asp His Tyr Arg Val Ser Arg			
	325	330	335	
	Ser Gln Val Glu Glu Ala Val Gly Arg Ile Asn Gly Arg Phe Gly Arg			
	340	345	350	
45	Met Gly Arg Pro Val Val His Tyr Leu His Arg Ser Leu Ser Lys Asn			
	355	360	365	
	Asp Leu Gln Val Leu Tyr Thr Ala Ala Asp Val Met Leu Val Thr Pro			
50	370	375	380	
	Phe Lys Asp Gly Met Asn Leu Val Ala Lys Glu Phe Val Ala Asn His			
	385	390	395	400
55	Arg Asp Gly Thr Gly Ala Leu Val Leu Ser Glu Phe Ala Gly Ala Ala			
	405	410	415	
	Thr Glu Leu Thr Gly Ala Tyr Leu Cys Asn Pro Phe Asp Val Glu Ser			
	420	425	430	

Ile Lys Arg Gln Met Val Ala Ala Val His Asp Leu Lys His Asn Pro
435 440 445

5 Glu Ser Ala Ala Thr Arg Met Lys Thr Asn Ser Glu Gln Val Tyr Thr
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His Asp Val Asn Val Trp Ala Asn Ser Phe Leu Asp Cys Leu Ala Gln
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10 Ser Gly Glu Asn Ser
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20 <220>
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35 <400> 4
accaaccagg tggaaatctgt ca 22

Patent claims

1. An isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the otsA gene, chosen from the group consisting of
 - 5 a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
 - b) polynucleotide which is complementary to the
 - 10 polynucleotides of a) or b),
 - d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),
- 15 the polypeptide preferably having the activity of trehalose 6-phosphate synthase.
2. The polynucleotide as claimed in claim 1, wherein the polynucleotide is a preferably recombinant DNA which is capable of replication in coryneform bacteria.
3. The polynucleotide as claimed in claim 1, wherein the
 - 20 polynucleotide is an RNA.
4. The polynucleotide as claimed in claim 2, comprising the nucleic acid sequence as shown in SEQ ID No. 1.
5. The DNA as claimed in claim 2 which is capable of replication, comprising
 - 25 (i) the nucleotide sequence shown in SEQ ID No. 1, or
 - (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or

- (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally
 - (iv) sense mutations of neutral function in (i).
- 5 6. The DNA as claimed in claim 5 which is capable of replication, wherein the hybridization is carried out under a stringency corresponding to at most 2x SSC.
7. The polynucleotide sequence as claimed in claim 1, which codes for a polypeptide which comprises the amino acid sequence shown in SEQ ID No. 2.
- 10 8. Coryneform bacteria in which the otsA gene is attenuated, in particular eliminated.
9. Coryneforme bacteria deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen [German Collection 15 of Microorganisms and Cell Cultures] under no. DSM 14041.
10. A process for the fermentative preparation of L-amino acids, in particular L-lysine, wherein the following steps are carried out:
- 20 a) fermentation of the coryneform bacteria which produce the desired L-amino acid and in which at least the otsA gene or nucleotide sequences which code for it are attenuated, in particular eliminated;
- 25 b) concentration of the L-amino acid in the medium or in the cells of the bacteria, and
- c) isolation of the L-amino acid.
11. The process as claimed in claim 10, wherein a Corynebacterium glutamicum strain deposited at the 30 Deutsche Sammling für Mikroorganismen und Zellkulturen

[German Collection of Microorganisms and Cell Cultures]
under no. DSM 14041 is employed.

12. The process as claimed in claim 10, wherein bacteria in which further genes of the biosynthesis pathway of the
5 desired L-amino acid are additionally enhanced are employed.
13. The process as claimed in claim 10, wherein bacteria in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly
10 eliminated are employed.
14. The process as claimed in claim 10, wherein the expression of the polynucleotide(s) which code(s) for the otsA gene is attenuated, in particular eliminated.
15. The process as claimed in claim 10, wherein the regulatory (or catalytic) properties of the polypeptide (enzyme protein) for which the polynucleotide otsA codes are reduced.
15
16. The process as claimed in claim 10, wherein for the preparation of L-amino acids, coryneform microorganisms
20 are fermented in which at the same time one or more of the genes chosen from the group consisting of
 - 16.1 the dapA gene which codes for dihydrodipicolinate synthase,
 - 16.2 the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase,
25
 - 16.3 the eno gene which codes for enolase,
 - 16.4 the tpi gene which codes for triose phosphate isomerase,
 - 16.5 the pgk gene which codes for 3-phosphoglycerate kinase,
30

- 16.6 the zwf gene which codes for glucose 6-phosphate dehydrogenase,
- 16.7 the pyc gene which codes for pyruvate carboxylase,
- 5 16.8 the mqo gene which codes for malate-quinone oxidoreductase,
- 16.9 the lysC gene which codes for a feed-back resistant aspartate kinase,
- 16.10 the lysE gene which codes for lysine export,
- 10 16.11 the zwal gene which codes for the Zwal protein is or are enhanced or over-expressed.
17. The process as claimed in claim 10, wherein for the preparation of L-amino acids, coryneform microorganisms are fermented in which at the same time one or more of the genes chosen from the group consisting of
- 15 17.1 the pck gene which codes for phosphoenol pyruvate carboxykinase,
- 17.2 the pgi gene which codes for glucose 6-phosphate isomerase,
- 20 17.3 the poxB gene which codes for pyruvate oxidase,
- 17.4 the zwa2 gene which codes for the Zwa2 protein,
- 17.5 the fda gene which codes for fructose 1,6-bisphosphate aldolase,
- 25 17.6 the hom gene which codes for homoserine dehydrogenase
- 17.7 the thrB gene which codes for homoserine kinase,

17.8 the panD gene which codes for aspartate decarboxylase

is or are attenuated.

18. Coryneform bacteria which contain a vector which carries parts of the polynucleotide as claimed in claim 1, but at least 15 successive nucleotides of the sequence claimed.

5 19. The process as claimed in one or more of the preceding claims, wherein microorganisms of the species Corynebacterium glutamicum are employed.

10 20. A process for identifying RNA, cDNA and DNA in order to isolate nucleic acids or polynucleotides or genes which code for trehalose 6-phosphate synthase or have a high similarity with the sequence of the otsA gene, wherein the polynucleotide comprising the polynucleotide sequences as claimed in claims 1, 2, 3 or 4 is employed 15 as hybridization probes.

21. Process as claimed in claim 20, wherein arrays, micro arrays or DNA chips are employed.

20

Abstract

The invention relates to an isolated polynucleotide comprising a polynucleotide sequence chosen from the group consisting of

- 5 a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- 10 b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the polynucleotides of a) or b), and
- 15 d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

and a process for the fermentative preparation of L-amino acids using coryneform bacteria in which at least the otsA gene is present in attenuated form, and the use of polynucleotides which comprise the sequences according to the invention as hybridization probes.

Figure 1: Plasmid pUC18otsA

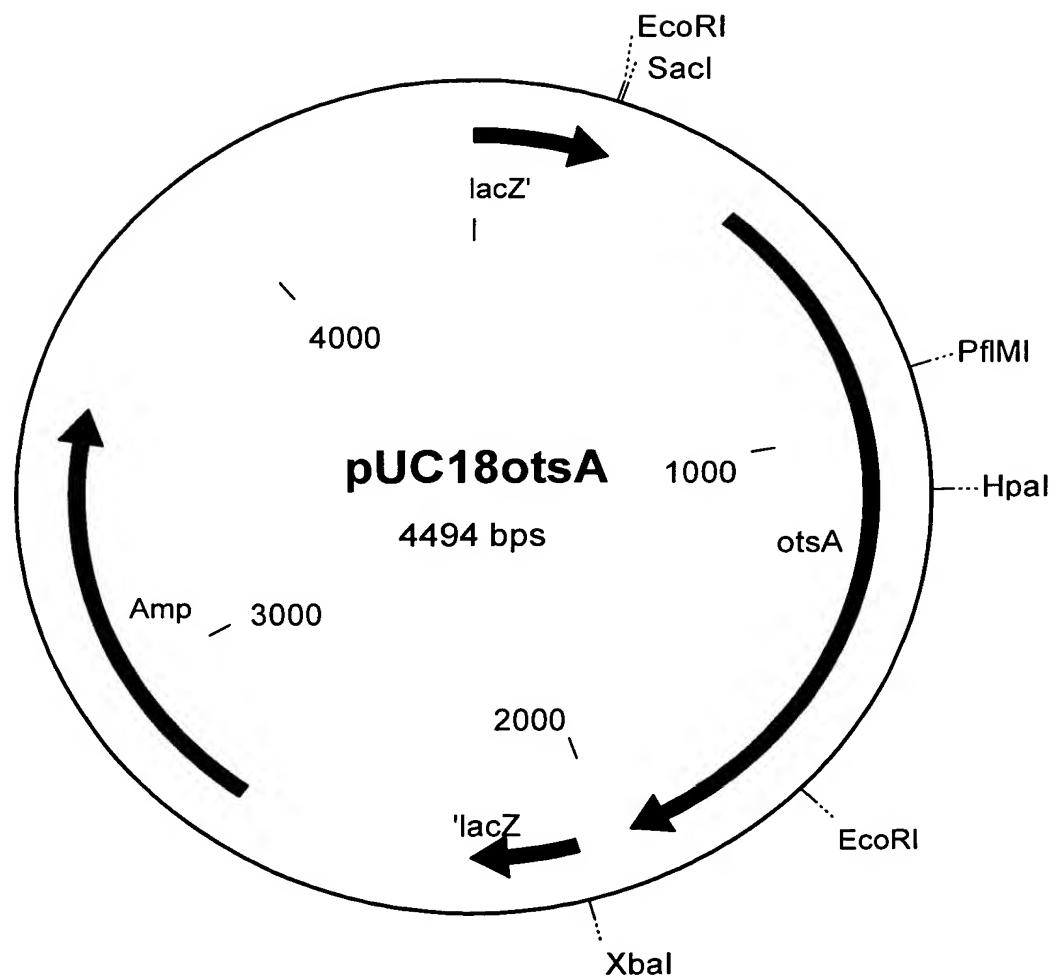


Figure 2: Plasmid pK19mobsacB Δ otsA